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NUTRITION REVIEWS

VOL. 18 July 1960 No. 7

FACTOR 3, SELENIUM AND VITAMIN E

The trails of nutrition research have often taken unexpected turns. One such turn occurred in 1957, when it was discovered that the element selenium plays a part as an essential micronutrient. This finding was made when selenium was identified as an integral constituent of Factor 3 (K. Schwarz and C. M. Foltz, *J. Am. Chem. Soc.* **79**, *3292 (1957)*). Factor 3, first described as an unknown agent protecting rats against dietary necrotic liver degeneration, has now been recognized to prevent a wide spectrum of deficiency diseases; for example, multiple necrosis in mice, muscular dystrophy and heart necrosis in mink, exudative diathesis in chicks and turkeys, stiff lamb disease and ill thrift in sheep, white muscle disease in calves, and liver dystrophy and muscle degeneration in pigs.

In some of these fatal diseases, Factor 3 has displayed a remarkable partnership with vitamin E and L-cystine. In fact, it was designated "Factor 3" to indicate that it was the third dietary agent found to be effective against liver necrosis. Meanwhile, the role of L-cystine has appeared in a new light; this sulfur amino acid owes its potency largely to a minute contamination with a trace of Factor 3-active selenium.

The Factor 3 problem started in 1939 as a side line to studies on bacterial growth factors. In order to investigate whether the growth factor H′ was essential to animals, a rigorously purified casein was produced (casein VI). Factor H′, isolated and identified as para-aminobenzoic acid (R. Kuhn and Schwarz, *Ber. Dtsch. Chem. Ges.* **74**, *1617 (1941)*), was not essential for rats. Yet, most of the animals on casein VI diets died after slightly more than a month from a sudden attack of liver necrosis. Against this deficiency disease, some natural products such as wheat germ, wheat bran, and whey were found to contain protective agents. Using prevention of liver necrosis as an assay, a study of these protective substances was begun. Through fractionation from wheat germs, tocopherol was identified as one of the main liver protecting agents (Schwarz, *Z. f. Physiol. Chem.* **281**, *109 (1944)*).

Liver necrosis on deficient diets had been observed by many others, especially in rats on low protein rations. In retrospect, it seems that the diverse diets used in all these studies were low in sulfur amino acids, and deficient in vitamin E and Factor 3. An early publication was that by T. E. Weichselbaum (*Quart. J. Exp. Physiol.* **25**, *363 (1935)*). He fed low cystine diets, observed "hemorrhages throughout the liver", and found that L-cystine prevented the damage. The protective effect of commercial L-cystine on liver necrosis was then more clearly established by F. S. Daft, W. H. Sebrell and R. D. Lillie (*Proc. Soc. Exp. Biol. Med.* **50**, *1 (1942)*).

That a third factor, other than L-cystine and vitamin E, was involved in the prevention of the disease became apparent early in our studies, but conclusive proof for the factor was obtained only after 1949. In Europe, it has been found during World War II that certain yeasts could be used as sole source of protein in diets which induced necrotic liver degeneration, but in the United States workers were unable to duplicate these findings with American yeasts. Some laboratories resorted to imported British bakers' yeast to produce the disease, until an American grown *Torula* yeast was found to be useful for the purpose (Schwarz, *Proc. Soc. Exp. Biol. Med.* **77**, *818 (1951)*). In the Section on Experimental Liver Diseases at Bethesda, a semisynthetic basal diet containing 30 per cent *Torula* yeast has been in use since then with consistent results. Con-

vincing proof for the existence of Factor 3 was obtained by supplementation of a few per cent of American brewers' yeast to this ration. The addition afforded complete protection. The same effect was elicited by small supplements of "vitamin-free" caseins, shown to contain Factor 3. The results indicated that liver necrosis was not caused by some hypothetical "necrogenic" influence; it simply constituted a deficiency of complex origin.

Comprehensive studies were carried out in the following years to determine the natural distribution of Factor 3, to devise suitable fractionation and isolation procedures for it, and to clarify its role in intermediary metabolism. Factor 3 was found to be widely but unevenly distributed in nature. Even in kidney, a potent natural source, it is present only in minute absolute amounts. Like many other micronutrients, Factor 3 is bound to its natural environment, apparently to protein, and cannot be extracted except after autolysis or hydrolysis. The factor is of low molecular weight, acidic, soluble in water and polar solvents like alcohol, but insoluble in lipid solvents.

In the course of the fractionation procedures, an enzymatic brewers' yeast autolysate, a digest of casein, and acid hydrolysates of dried, fat-extracted pork kidney were used as source materials. Sizeable batches of raw material had to be worked up, and several thousand rat assays with Factor 3 fractions were run before highly potent, microcrystalline, but still impure Factor 3 fractions could be obtained in milligram quantities. These displayed a characteristic, garlic-like odor, which suggested the presence of selenium.

Analyses of Factor 3 preparations of greatly divergent activities revealed that the biopotency was correlated to the selenium content. Then it was realized that rats can utilize known compounds of selenium very effectively. It would be erroneous, however, to equate Factor 3 simply with selenium. Large differences in potency exist between different selenium compounds, and, per atom of selenium, Factor 3 is more potent than any other compound tested thus far.

It has occurred repeatedly in the field of nutrition that, with the identification of a new dietary agent, several other seemingly distant questions found their solution. This has also been the case with Factor 3. In 1955, M. L. Scott and collaborators adapted the *Torula* yeast ration to the production of vitamin E-deficiency in chicks. The diet caused growth failure, exudative diathesis, and death (Scott *et al.*, *J. Nutrition* **56,** *387* (*1955*)). It was ascertained during the same year that Factor 3 effectively prevented this disease in chicks, just as it prevented dietary liver necrosis in the rat. Scott *et al.*, at Cornell, as well as J. G. Bieri and G. M. Briggs at Bethesda, assayed our Factor 3 concentrates from kidney, brewers' yeast and casein. Between both species, a close parallelism was found in the effectiveness of Factor 3 preparations, some crude, and some several hundred-fold concentrated. On the basis of these results, a group at Lederle Laboratories started to use the chick assay and the *Torula* yeast diet for an investigation into the nature of Factor 3.

After the discovery of selenium in Factor 3 was made in March, 1957, in our purification project using the rat assay, a cooperative study was arranged between Cornell and Bethesda to test the potency of selenium compounds in chicks. Selenite and selenocystathionine were highly potent; elementary selenium protected at very high levels (Schwarz, J. G. Bieri, G. M. Briggs and Scott, *Proc. Soc. Exp. Biol. Med.* **95,** *621* (*1957*)). The Lederle group had observed that alkaline but not acidic ashes of crude Factor 3 fractions retained approximately 20 per cent of the activity. They independently described the effect of selenite against exudative diathesis (E. L. Patterson, R. Milstrey and E. L. R. Stokstad, *Ibid.* **95,** *617* (*1957*)).

Selenium is one of the rarer elements, with properties very similar to those of sulfur. The two often occur together, and there is a

rather complete analogy between the inorganic and organic compounds of both elements. Selenium analogues of the sulfur amino acids have been found in biological materials, such as proteins of seleniferous plants, and can be synthesized. With respect to biopotency against liver necrosis in rats, selenium compounds can be divided into three main categories (Schwarz and C. M. Foltz, *J. Biol. Chem.* **233,** *245 (1958)*). Elementary selenium and certain inorganic and organic compounds are practically inactive. A second group affords protection at levels supplying a few micrograms of the element per 100 g. of diet. Their 50 per cent effective dose (ED_{50}) is about 2 to 3 micrograms per cent selenium (0.02 to 0.03 p.p.m.). Most inorganic selenium compounds, such as selenate and selenite, many organic selenium compounds, and also the selenium analogues of cystine, cystathionine and methionine belong to this category. It appears that the rat and the chick can utilize selenium from all these sources with equal efficiency.

The third category, comprising substances of higher biopotency, is small. Thus far only four synthetic organic compounds were found to be more active than selenite. None of them, however, reaches the biopotency of Factor 3. The latter has an ED_{50} of about 0.7 micrograms per cent selenium, as established by standardization of various Factor 3 preparations in the animal assay and determination of their selenium contents by radioactivation analysis, colorimetric assay and micro-analytical methods. Radioactivation analysis is the method of choice; it has a sensitivity of 0.01 micrograms selenium per gram of material. The method consists of the production of radioactive isotopes by neutron bombardment and subsequent determination of the element in question by carrier analysis. A daily dose of 0.1 microgram of Factor 3-selenium fully protects rats against death. It also enhances growth significantly. This requirement is in accordance with the amounts of selenium found in natural nutrients, which are of the order of 0.05 to 10 micrograms per gram of dry matter.

Selenium estimation, however, does not result in an estimate of Factor 3 activity, since some nutrients contain the element in a form which is biologically inert. At the moment the animal assay is the only feasible method for the determination of the Factor 3 content of dietary ingredients.

One atom of selenium, in form of Factor 3, affords as much protection as 700 to 1000 molecules of vitamin E in preventing dietary liver necrosis in the rat, multiple necrotic degeneration in the mouse, and exudative diathesis in the chick. L-Cystine, in turn, possesses only a very small fraction of the potency of tocopherol; to elicit 50 per cent protection, about 0.4 per cent must be added to the diet. The biopotency of Factor 3 is so high that a trace contamination of one atom of Factor 3-selenium among 355,000 atoms of sulfur could account for the biological potency of the sulfur amino acid. This amount is indeed found in commercial L-cystine, which is manufactured from horn, hoofs and hair and contains up to 2 micrograms of selenium per gram. Procedures which remove the selenium from the cystine also eliminate the biopotency (Schwarz, J. A. Stesney and Foltz, *Metabolism* **8,** *88 (1959)*).

Methionine and cystine, when clearly free from traces of selenium, do not prevent liver necrosis. However, they delay its onset when supplied at levels exceeding the requirement for growth. The delay is the result of a sparing effect on the requirement for vitamin E. The sulfur amino acids reduce the vitamin E requirement to only one tenth of the level normally necessary for protection. Thus they exert a modulating effect on the condition, but in essence dietary liver necrosis is the result of the simultaneous lack of Factor 3-selenium and vitamin E.

While the lack of Factor 3 alone, or that of vitamin E by itself, produces often relatively mild chronic diseases, the simul-

taneous deficiency of both leads invariably to acute, impressive tissue damage and to death. The pathological signs of what in the past was thought to be vitamin E-deficiency range from mild afflictions, such as resorption sterility or testicular atrophy, to fatal changes in liver, kidney, heart, muscles, lungs, pancreas, brain and spinal cord. A number of these diseases, hitherto attributed solely to vitamin E, are of dual origin. The presence or absence of Factor 3-active selenium determines the fate of an animal on a vitamin E-deficient diet.

Regardless of this interrelationship, selenium neither spares vitamin E, nor does it substitute for it. It is an essential dietary constituent in its own right. In rats provided with tocopherol, Factor 3-deficiency causes a distinct disease entity comprising lack of growth, muscular wasting, adrenal atrophy and pancreatic dystrophy. In the chick, Factor 3-active selenium has a growth effect independent of vitamin E. And in ruminants, selenium begins to play an important practical role in "stiff lamb disease", and in white muscle disease in calves (O. H. Muth, J. E. Oldfield, L. F. Remmert and J. R. Schubert, *Science* **128,** *1090 (1958)*; J. F. Proctor, D. E. Hogue and R. C. Warner, *J. Animal Sci.* **17,** *1183 (1958)*).

These diseases, consisting of muscular dystrophy, calcification, and heart muscle degeneration, are enzootic in the northwestern United States, New Zealand, Scotland, Sweden, Finland, etc. In some areas, they are affected only little by vitamin E. A single dose of 1 mg. of selenite selenium greatly increases the number of live-born lambs, prevents congenital white muscle disease, as well as field outbreaks of white muscle disease in lambs, and eliminates post-weaning "unthriftiness", probably the most important cause of economic loss in this industry in New Zealand (C. Drake, A. B. Grant and W. J. Hartley, *New Zealand Vet. J.* **8,** *4 (1960)*).

In principle, then, one must separate clearly three groups of diseases: those caused purely by vitamin E deficiency, which are not influenced by Factor 3-selenium even in large excess (resorption sterility in rats, encephalomalacia in chicks); those caused purely by Factor 3-selenium deficiency, which are not affected by vitamin E; and those caused by simultaneous lack of both factors. To the last category belong dietary liver necrosis in the rat, multiple necrosis in the mouse, heart and peripheral muscular dystrophy in the mink, liver necrosis and muscular dystrophy in the pig, and exudative diathesis in chicks and turkeys.

It is common medical knowledge that a specific damage, for example an infection, shows different sites of predilection in different species. The same applies to deficiency diseases. Necrotic liver degeneration is only a special instance of a widespread injury involving many other tissues as well. The liver is the site of predilection in the rat. In other animals, damage to other organs is more predominant. The heart, for instance, is involved in mice, mink and sheep, the peripheral muscles degenerate in most species, and kidneys, pancreas, lungs, adrenals, etc., are more or less severely damaged in most mammals, while exudative diathesis, probably due to hypoproteinemia, prevails in birds.

The involvement of so many tissues may indicate that a function of fundamental importance to metabolism breaks down. Studies with liver slices from rats on the necrosis-producing diet showed that a metabolic defect, respiratory decline, is present one to two weeks before actual necrosis occurs. Such liver slices cannot maintain normal oxygen consumption. Addition of vitamin E to the diet, or the intravenous injection of the vitamin ten to 30 minutes prior to extirpation of the liver will completely prevent the breakdown. Factor 3-selenium affects respiratory decline independently, but to a lesser degree. It seems that the impairment which leads to liver necrosis involves energy metabolism.

A working hypothesis to explain the

synergistic partnership of vitamin E and selenium postulates that the two have independent catalytic effects in alternate pathways of metabolism (Schwarz, *Liver Function, p. 509. Am. Inst. Biol. Sciences, Washington, D. C. (1958)*). This would explain why the absence of both is necessary for the development of most of these injuries.

In the past, selenium has received a great deal of attention for its poisonous properties, and hardly any for beneficial effects. Thus S. F. Trelease and O. A. Beath have stated in their outstanding review on selenium poisoning that "as far as we know it is toxic, but never beneficial to animals or man" (Trelease and Beath, *Selenium, New York (1949)*). The toxicity of selenium compounds varies greatly with their chemical constitution, and it is dependent on the nutritional status of the animal. The minimum chronic toxic dose of selenite-selenium is approximately 300 to 400 micrograms per 100 g. of diet (3 to 4 p.p.m.). If this level is compared to that which protects against Factor 3-deficiency, we see that selenium is relatively not more toxic than other dietary constituents. The ratio between effective dose and toxic dose is of the order of 1 to 100, which compares favorably with the therapeutic indices of amino acids, salts, and almost all other important nutrients.

The discovery of Factor 3 and the detection of the biological role of selenium serve to illustrate the point that there may still be new essential dietary agents left to be explored through animal experimentation in conjunction with modern methods of biochemistry. One may be tempted to think that a factor with protective qualities against serious lesions in so many species may play a role in man. Exacting and critical studies on human diseases are needed in areas indicated by animal experiments in order to substantiate this conclusion.

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PROTECTIVE EFFECT OF MILK AGAINST BONE STRONTIUM-*90* ACCUMULATION

Although the level of strontium-90 in milk has been gradually increasing, the high calcium content of milk makes it one of our best protective foods for the prevention of strontium-90 accumulation in humans.

Since milk and milk products make up a large part of the diet of Americans and especially the diet of children, the reports released by the U. S. Public Health Service of gradually increasing levels of strontium-90 in milk have led to increasing concern on the part of both professional and lay persons. Although there has been interest and research into the possibility of removing strontium-90 and other radionuclides from milk and into the possibilities of milk substitutes, a consideration of the significance of strontium-90 in milk and milk products in terms of human accumulation of this source of radiation seems most opportune.

B. L. Larsen (*J. Dairy Sci.* **43,** *1 (1960)*) has reviewed the currently available information on strontium-90 in milk and the effect of milk on strontium-90 accumulation in the human popu!ation. His survey gives strong support to the claim that milk is still our most satisfactory protective food, whether in terms of supplying needed nutrients or in terms of preventing strontium-90 accumulation in the bones of our population.

Strontium-90 originates primarily in fallout, and the factors which influence fallout are of great concern to all of us. The three most important factors in fallout are (1) the size and location of nuclear explosions made to test nuclear weapons, (2) the winds of the various latitudes and (3) the annual rainfall in any given area. E. A. Martell (*Science* **129,** *1197 (1959)*) has presented data indicating that the amount of fallout at the forty-fifth latitude north has been approximately five times that of fallout at the forty-fifth latitude south and 15 times that at the equator. M. Eisenbud, in considering this same problem (*Ibid.* **130,** *76 (1959)*), has indicated that the bulk of the fallout lies between the fortieth and fiftieth latitude, both north and south, but that the fallout in the north latitudes is three times that in the south.

The increasing rapidity with which strontium-90 fallout has developed has led to consideration of the strontium-90 present in rainfall. At present it appears that cistern water may be giving more strontium-90 than is secured from foods, and recent data indicate that cistern water is now averaging from 3 to 5 micro microcuries of strontium-90 per liter.

With the accumulation of data indicating a much higher rate of fallout in the northern latitudes, it is of particular interest that J. L. Kulp, A. R. Schulert and E. J. Hodges (*Science* **129,** *1249 (1959)*) have reported that accumulations of strontium-90 in human bones are similar in both the northern and southern hemispheres. That there has been a continuing increase over the past ten years in the strontium units (a strontium unit, "S.U.", is one micro microcurie of strontium-90 per gram of calcium) is revealed by their data, which shows an increase from a value of approximately 0.12 S.U. to approximately 0.19 S.U. in human bones.

A number of factors appear to influence this increase in strontium-90 in human bones. In the southern hemisphere, vegetables and cereals supply most of the calcium in the diet as opposed to milk in the northern hemisphere. Moreover, there is a high rainfall in tropical regions and a relatively low calcium availability in tropical soils. Finally, there is a global flow of food products, such as wheat and dried milk, which tends to equalize the types of diet and the levels of strontium-90 in the diet in both hemispheres. However, it should be pointed out that the highest levels of bone calcium were found in those people on rice diets in the southern hemisphere. Thus, despite the apparent equalization of strontium-90 in the bones of the human population, it appears probable that the populations receiving dietary calcium from milk and milk products are accumulating relatively lower levels of strontium-90 in their bones than those consuming primarily vegetable and cereal products.

As of January 1958, adults had an average bone strontium-90 accumulation of 0.19 S.U. while children had approximately five times this amount. It is estimated that, with the present strontium-90 burden of the atmosphere and soil, a maximum value or equilibrium in children will be reached in 1966 with approximately 4.0 S.U. in the bones.

These values must be viewed against a background of what is considered a hazard. The National Committee on Radiation Protection in reviewing the permissible burdens of strontium-90, cesium-137 and iodine-131 for workers in atomic plants have set the permissible strontium-90 bone level as 2 microcuries and the cesium-137 level in muscle as 50 microcuries. On the other hand, the National Committee on Radiation Protection has arbitrarily set the levels for populations in the vicinity of atomic installations at one-tenth of these levels. By comparison then, workers in atomic plants have a permissible burden of 2000 S.U. whereas the neighbors to these plants have a permissible level of 200 S.U. The permissible level for the population as a whole has been set at 100 S.U., compared with 4 S.U. as the predicted level for children in 1966

(*National Bureau of Standards Handbook* **69** (*1959*)).

The reports on strontium-90 in milk indicate a steadily increasing value as more and more fallout returns to the soil from the atmosphere, yet in 1958 at the cessation of nuclear testing, values for milk from many cities around the country indicated average values ranging from 2 to 16 S.U. per liter.

Although milk has been considered in greater detail, increasing attention has been given more recently to the strontium-90 accumulation in cereals, vegetables, fruits and other items of the diet. The vegetable and cereal strontium-90 values have led to a realization that, especially in the adult diet, these products may actually be supplying from five to 20 times the level of strontium-90 supplied in the diet by milk. For example, the 1958 wheat crop showed from 64 to 330 S.U., and in the August 14, 1959 issue of the *Washington Post* there was a report that the Food and Drug Administration had found strontium-90 in alfalfa at levels ranging from 136 to 806 micro microcuries of strontium-90 per kilogram.

These values, which have been reported variously as micro microcuries of strontium-90 per kilogram and as strontium units (which take into consideration the calcium level), may be misleading unless the widely varying calcium content of vegetable products is recognized.

The reporting of strontium-90 as micro microcuries, rather than as strontium units, places major emphasis upon the level of strontium-90 in the diet rather than on the strontium-90 that will accumulate in the bones. Milk may contain ten times as much calcium as potatoes, but, if milk and potatoes contain the same strontium-90 level, a diet of potatoes will give a bone deposit of strontium-90 that will be five to ten times that from a diet of milk.

The only satisfactory way to evaluate a diet must be in terms of both calcium content and strontium-90 level. Because most foods contain less than 1 g. of calcium per kilogram, the micro microcuries of strontium-90 per kilogram will be lower than the strontium units. This may lead to a tendency to eliminate those foods with highest strontium-90 content, not recognizing that if calcium levels are thus ignored, the calcium level of the diet will be reduced and deposition of strontium-90 will increase.

On the basis of strontium units, calculated from a report of the levels found in dietary items in those areas influenced by uranium refinery waste (*U. S. Public Health Service Report. Survey of Interstate Pollution of the Animus River* (*1959*)), it was noted that milk contained 5 S.U., cabbage 210 to 682 S.U., potatoes 37 to 50 S.U., peas 1137 S.U., sweet corn 34 to 200 S.U., apples 110 S.U., lettuce 86 to 4420 S.U. Interestingly enough, alfalfa, wheat and oats in these same regions contain less than 10 S.U.

All of these results pose very serious problems in the effort to determine the effects of long-term, low-level radiation on humans. If the present accumulation is negligible, then there is no problem. Certainly current evidence is that the present and projected levels of accumulation are minor compared to natural sources of radiation such as potassium-40 and carbon-14, or compared to the irradiation from medical practice. However, until we have more definite data, the only reasonable procedure is to keep strontium-90 accumulation to a minimum.

Efforts to reduce strontium-90 accumulation in the bone must take into consideration the fact that the assimilation and hence the incorporation into bone of strontium-90 from milk is decreased by an increase in the calcium level of the milk, or of the diet as a whole. A source of calcium which did not contain strontium-90, such as mineral sources of calcium, would then have a real advantage. Reducing the strontium-90 which comes from other foods should also be considered, since it appears that there is less discrimination against strontium-90 in low-calcium vegetable and cereal items. It certainly is probable that as the strontium-90

level of vegetable items of the diet increases, milk will provide the greatest protective action because of the discrimination by the body of the cow and the human body against strontium in favor of calcium.

This discrimination may be even more important if results recently reported by A. E. Sobel, S. Nobel and P. A. Laurence (*Chem. Eng. News* **37,** *42 (1959)*) are correct. Their results indicate that strontium-90 may not enter into the crystalline structure of bone and, therefore, would be subject to exchange with calcium, favored by high calcium- and vitamin D-containing diets. This would explain the much more rapid removal of strontium than of calcium from bone observed by others (A. R. Schulert *et al., Int. J. Applied Radiation & Isotopes* **4,** *144 (1959)*).

The data of Larsen emphasize the many problems to be considered in any evaluation of the role of milk in strontium-90 accumulation in man. More particularly, the data emphasize that milk is still the best protective item in the human diet. The need for more knowledge about the strontium-90 content of other items in the diet and, particularly, about the effects of diet variation upon strontium-90 accumulation certainly appears crucial. However, in the light of this review of the role of milk, it appears that because of its protective action, a more satisfactory reduction in terms of strontium-90 accumulation in bones may be obtained by doubling the calcium level of the diet rather than by removing as much as 50 per cent of the strontium-90 from the milk.

MAGNESIUM BALANCE IN ALCOHOLICS

Using metabolic balance techniques, alcoholic patients were found to retain more magnesium than non-alcoholic hospital patients used as controls. This was interpreted as indicating that the alcoholic patients were deficient in magnesium.

While magnesium has been known for some time to be an indispensable nutrient, its daily requirement for man is not known. Dogs and rats fed diets grossly deficient in magnesium develop a clinical picture which includes vasodilation, cardiac arrhythmia, exaggerated response to external stimuli, spasms and convulsions. If the deficiency is not relieved then death ensues. In the case of man, magnesium deficiency has been associated with tremors of the extremities, choreiform and athetoid movements of all muscles, convulsions, sweating and degrees of mental clouding which may progress to delirium and death (*Nutrition Reviews* **18,** *72, 101 (1960)*). Since magnesium is widely distributed in most foods, an individual eating a general mixed diet does not develop a deficiency of this substance.

Chronic alcoholics as a group possess a wide spectrum of nutritional deficiences because they obtain their caloric needs mainly from drinks which are deficient in most nutritional substances except alcohol, water and carbohydrate.

Recently, R. J. McCollister, E. B. Flink and R. P. Doe (*J. Lab. Clin. Med.* **55,** *98 (1960)*) studied the status of magnesium in a group of 11 individuals. Using the metabolic balance technique, they first investigated the level of magnesium in a group of seven non-alcoholic men, hospitalized for treatment of peptic ulcer, who served as controls. Five of these men, fed diets consisting of milk or half milk and half cream plus supplementary foods such as toast or eggs, were studied for periods ranging from four to eight days.

Their magnesium intake varied from 87.9 to 154 mEq per period (17 to 24 mEq per day) with an average value of 113.9 mEq, while their urinary output varied from 17 to 104 mEq per period with the high and low values corresponding with the high and low

intakes. Their fecal excretion was quite constant, ranging between 59 and 82 mEq per period. The magnesium balance for these individuals varied from +17 to −27 mEq per period, equivalent to +3.4 to −4.5 mEq per day.

Two men of the same type were maintained on a similar diet and, in addition, were given magnesium sulfate intramuscularly at the rate of 8 or 16 mEq per day for a six-day period. The total magnesium intake for these two patients was 123 and 161 mEq per period (20 to 27 mEq per day). Their urinary output was increased above those who did not receive the injections of magnesium and averaged 181.8 mEq. per six-day period. Both individuals showed a positive magnesium balance of 4.8 and 10.0 mEq per period (1.3 and 1.8 mEq per day).

Six alcoholic patients were treated with the diet alone for periods of six to ten days. They had an average magnesium intake of 208 mEq per period (17 to 31 mEq per day). Under these conditions their magnesium balance varied from +39 to +151 mEq per period (from 6.0 to 16.8 mEq per day). Five additional alcoholic patients treated with the diet plus magnesium had intakes ranging from 97 to 350 mEq per period (all periods were six days except for an eight-day period for the last individual). This intake amounted to 16 to 43 mEq per day. These patients had positive magnesium balances of 35 to 170 mEq per period, ranging from 6 to 21 mEq per day, with the largest balance in the patient who received the largest intake.

These investigators concluded that, since the alcoholic patients on the magnesium refeeding program retained considerably more magnesium than did the hospital patients used as controls, the magnesium stores of the alcoholic patients were grossly depleted. Also they noted that there was little change in the serum magnesium levels even after the patient showed a very good positive magnesium balance.

While in general these investigators reported that the alcoholic patients showed good positive magnesium balances after magnesium administration, it should be noted that, even before they were given intramuscular magnesium, the alcoholic patients had a much larger intake of the element than did the hospital control patients (208 mEq compared to 113 mEq). Thus one is not sure whether the non-alcoholic and supposedly non-depleted patient might show a much better positive magnesium balance had he received a greater magnesium intake.

Since magnesium deficiency also has been reported to occur in patients maintained on magnesium-free parenteral fluids (glucose or sodium chloride but not protein hydrolysates since they contain magnesium), magnesium deficiency may also occur in patients ill with diarrhea, vomiting, many types of malnutrition and some osteolytic bone lesions, as well as in patients who are taking very little food orally. Thus the possibility that magnesium deficiency occurs in a large number of patients may be quite real. More investigations in this field may lead to better care.

PROTEIN MALNUTRITION IN SOUTH INDIA

A combination of poverty, ignorance and disease has resulted in a general deficiency in calories and a specific deficiency in protein for most of the children of South India.

From 1955 to 1957 the World Health Organization and the Indian Council of Medical Research sponsored a survey of protein malnutrition in the South India states of Kerala, Madras, Mysore and Andhra Pradesh. Some of the preliminary results obtained in this survey have already been published (*Nutrition Reviews* **15,** *169 (1956)*; S. G. Srikantia, S. Sriramachari and C. Gopalan, *Ind. J. Med. Res.* **46,** *121 (1958)*;

Gopalan, *Ibid.* **46,** *317 (1958)*). A more complete summary of the results has recently appeared (K. S. Rao, M. C. Swaminathan, S. Swarup and V. N. Patwardhan, *Bull. World Health Org.* **20,** *603 (1959)*).

The region considered in the study covers 248,130 square miles with a population of 94.8 millions, ranging from 261 per square mile in Mysore to 908 in Kerala. Over 80 per cent of the population is agricultural, less than half of which till their own land. The principal foodstuffs raised in the region are cereals (mainly rice and millets) with smaller amounts of pulses, tapioca, milk, fish and vegetable oils. The per capita production of calories is 1842 per day including 45 g. of protein.

The 4536 children studied were divided about equally between urban and rural areas, but almost all were from the less-favored social groups. More than half the families had incomes of less than 50 rupees per month (1 rupee is about 21 cents) and less than 1 per cent of them had incomes greater than 150 rupees per month. Their living conditions, whether in city or country, were inadequate and unsanitary.

The main foods consumed by most of the families studied consisted largely of cooked cereals (largely rice or millets) and a spiced soup containing pulses and vegetables whenever available. Meat was available very infrequently and fish and milk, when produced, were largely sold in the cities so that few of these protein-rich foods were available for consumption at their source.

The children themselves had few of the advantages of modern medical practice or even of the most rudimentary cleanliness and hygiene. Not only poverty, but also ignorance and tradition were responsible for these conditions, which were so general that disease was commonplace, rarely treated effectively and probably responsible in part for the nutritional disorders which were the subjects of this study.

Weaning customs differed in the several states, but in general, about 50 per cent of the babies were weaned at 18 months and 80 per cent at 24 months. Except in Hyderabad (Andhra Pradesh), most of the babies received some supplementary food at one year, usually cereals, rarely animal milk (except in Mysore). Although the protein content of breast milk of Indian mothers was found to be in the normal range, the protein and caloric intakes of children between six and 36 months were considerably below the FAO recommendations, and it is in this period that kwashiorkor is most frequently found. Up until six months the mothers' milk was apparently adequate, while after three years of age the children consumed an average diet.

Clinical examination revealed that most of the symptoms associated with protein malnutrition were common. One quarter of the children examined had had recurrent diarrhea while 2 to 5 per cent had had edema. Over 60 per cent had dry inelastic skin, if not the typical dermatosis of kwashiorkor. Sparseness and dispigmentation of the hair, "moon face" and hepatomegaly were common in all regions. As many as 38 cases of frank kwashiorkor were found in the homes, although most cases had been removed to the hospital (where this disease accounts for 5 to 16 per cent of pediatric admissions). However, it is interesting that the average serum hemoglobin and serum protein in children with these symptoms were no lower than in the children without apparent symptoms. Most of the children appeared to have some degree of emaciation, and heights and weights of the children examined were well below the lowest percentiles for American children of the same age groups. It was thus apparent that the Indian children were suffering not only from a deficiency of protein but also of calories and, probably, of vitamins.

To bring the problem into focus, the authors state that by the most conservative estimates there are at least 120,000 cases of kwashiorkor in the region under study at any one time, while millions are suffering

from milder forms of protein malnutrition. They list three major causes for this sad state of affairs: poverty, with most families so poor that their entire incomes could not provide adequate nourishment; ignorance, which prevents the use of protein-rich foods for children even when available and especially when needed; infection and infestations, which often trigger the attacks of kwashiorkor and which aggravate an always marginal state of nutrition.

The solution to these problems is not going to be simple or rapid. However, research of the type reported by the present authors demonstrates that adequate protein nutrition in India can be achieved by supplementation of the usual high-cereal diet with high-protein protective vegetables such as the pulses and, where possible, with animal products such as milk and fish. The importance of this solution is that no new or expensive foods need be introduced to an unwilling public. The main problem, then, appears to be one of education, to convince the people that nutritional health is desirable and to show them how to achieve it and, at the same time, improve their economic status.

RECOMMENDED DIETARY ALLOWANCES

The newly revised allowances are discussed by appropriate representatives in terms of their uses and applicability to the general population, the armed services and the food industry.

The Recommended Dietary Allowances were first published by the Food and Nutrition Board of the National Research Council in 1943. The latest revision (*National Academy of Sciences-National Research Council Publication No. 589, Washington, D.C. (1958)*) represents the fourth since its original presentation. Recently, R. E. Shank has discussed the most significant changes introduced in this new edition (*Am. J. Pub. Health* **49,** *1001 (1959)*).

Prior to the establishment of Recommended Dietary Allowances, the usual standards met minimal needs. The allowances, however, sought higher objectives, being designed to maintain good nutrition in healthy persons in the United States under current conditions and to cover nearly all variations of requirements for nutrients in the population at large. They are, however, not considered adequate to meet the additional requirements deriving from disease or for nutrient repletion, but are more generous than is often practicable when large groups must be fed under conditions of limited food supply or economic stringency.

It was with the foregoing in mind that the following changes were made. The first of these relates to "reference" individuals. With the previous (1953) revision, the reference individuals had been changed to correspond with conditions which were specified by the Food and Agriculture Organization but which did not correspond to conditions prevailing in the United States. For instance, the weight of the reference man was 65 kg., whereas the average adult man, aged 25, is approximately 70 kg. in this country. Moreover, the FAO specified a mean external temperature of 10°C, whereas the average temperature under which most Americans live seems closer to 20°C. Accordingly, the reference man was changed to 25 years of age, 69 inches tall, weighing 70 kg. (154 pounds), living at a mean external temperature of 20°C and engaged in moderate physical activity. Similar changes were made for the reference woman.

The new revision also contains tables providing calorie allowances for persons of varied size and age. Available data substantiate the decreases in caloric require-

ments with advancing age. Accordingly, calorie allowances given for age 45 are now 6 per cent less and for age 65 are 21 per cent less than for age 25.

Estimates for calorie allowances were also modified with respect to climate. A 5 per cent decrease for each increase in temperature of 10 degrees was retained, but an allowance of only 3 per cent is given for each 10 degree decrease in external temperature. This change is based on the evidence that persons usually protect themselves from the cold by use of warm clothing and by living and working in heated buildings.

It is recognized that people engaged in heavy labor will have enhanced food requirements but that this requirement seldom need be increased more than 25 per cent above the standard allowance. On the contrary, the most important problem is thought to be the large proportion of persons who expend very little physical energy. Fortunately, according to R. E. Shank, there is renewed interest in assessing energy expenditures in common activities and employment. He suggests that perhaps a later revision of the Recommended Dietary Allowances will be able to offer more satisfactory modifications of the caloric requirements of physical activity.

The most time and effort was devoted by the Board to the consideration of protein allowances, and a new approach was sought in the light of world-wide research interest in protein requirements. However, the Board retained the approach to protein allowances utilized in earlier editions as well as the long-accepted value of 1 g. protein per kilogram of desirable weight. It was agreed that this allowance is not excessive and probably includes a reasonable margin of safety to meet variations in the consumer protein quality. A provision is made for an additional intake of 20 g. protein per day above the basal allowance during the last half of pregnancy and 40 g. per day for lactating women. No allowance was stated for infants during the first months of life because of the insufficiency of data. This deficiency of data should serve as an inducement for heightening research efforts in this area.

Despite evidence of a wide variation in calcium requirements, as observed in balance studies, the allowance of 800 mg. of calcium per day for adults (excluding pregnancy and lactation) was continued. Iron allowances were revised downward from 12 mg. iron per day to 10 for the adult male, as compared to 12 mg. for the adult female. This recognizes the lower rate of iron loss in the male as compared to the female during the years of menstruation.

Vitamin allowances were little changed and any alterations were based primarily on the changes introduced in the size of the reference man and woman. The major exception was that of the niacin allowance which was re-evaluated in terms of the quantity of the preformed vitamin available in mixed diets plus that derived from its precursor, tryptophan.

Dr. Shank emphasizes that the allowances were planned for healthy, moderately active persons. The presence of acute or chronic illness may modify requirements markedly. In order to serve as a guide for planning adequate diets for population groups, allowances must be weighted not only in terms of distributions of ages, sexes and activity categories but also for losses due to storage, cooking and serving.

RDA in Military Nutrition

One of the most logical applications of the Recommended Dietary Allowances is in the maintenance of the military. T. E Friedemann, H. F. Kraybill and C. F. Consolazio (*Am. J. Pub. Health* **49**, *1006 (1959)*) discuss this subject in terms of (1) the Army's basic dietary standard and (2) the soldier's actual nutrient intake. They point out that the Quartermaster General is responsible for the choice of foods, the formulation of menus and the administration of the feeding program in the Army. Since July 1941, the procurement and serving of food has been prescribed in a Master Menu published six months in advance and used thoughout the

Army. This is based on the Recommended Dietary Allowances as modified by the special requirements of troops under various operational conditions.

The Army basic standard sets the minimum intake of the physically active soldier in a temperate climate as: 3600 calories, 100 g. protein, 700 mg. calcium, 5000 i.u. vitamin A, 75 mg. vitamin C, 1.7 mg. thiamine, 2.0 mg. riboflavin and 16 mg. niacin. During 1957 the average edible nutrients provided by the Master Menu were actually approximately 4195 calories, 131 g. protein, 199 g. fat and 470 g. carbohydrate. Vitamins and minerals were provided well above the recommended intake.

In surveys conducted in four training camps it was found that the average of nutrients consumed in the mess and from other sources was 4265 calories, 131 g. protein, 201 g. fat and 484 g. carbohydrate. The average caloric requirement for maintenance of weight in very active young soldiers was 4066 calories, while the average for moderately active soldiers was 3175 calories.

Of interest is the high fat content of the the Army diet. Assuming that 1 g. of fat yields 9 calories of metabolizable energy, the 199 g. of fat in the 1957 menu furnished 43 per cent of the total daily calories of the ration. This must be considered high, but it is pointed out that it is within the range of fat-calorie intake of the high-income civilian groups in this country.

RDA in the Food Industry

According to H. E. O. Heineman and E. Bennett (*Am. J. Pub. Health* **49,** *1013 (1959)*), the Recommended Dietary Allowances have had a considerable influence on the food industry, awakening it to the desirability of staff personnel scientifically trained in nutrition and allied fields. This personnel has, for the most part, upgraded the standards by which the food industry operates. The Recommended Dietary Allowances are a useful guide for the food industry, and have spurred research to seek crops of highest nutrient quality, to develop better processing methods, and to find packaging materials and marketing systems which will maintain original nutrient values until consumption. They have helped establish the level of fortification of some of our foods, such as vitamin D-enriched milk and enriched bread and flour. They have also functioned as a barrier against low and useless additions of nutrients, although credit must also be given to the control exercised by the Food and Drug Administration.

The authors believe that the Recommended Dietary Allowances can act as an important checkrein despite intense competition in the food industry. Emanating from an unbiased group of scientists, they are a carefully controlled compilation constantly reviewed in the light of new research. Thus they can help the food industry maintain stability and balance in the midst of new, confusing and sometimes conflicting research data on specific nutrients and nutrient needs. It is to be hoped that the Recommended Dietary Allowances will be even more generously and wisely used by the food industry in the future.

EFFECT ON SERUM CHOLESTEROL LEVELS OF REPLACING MILK FAT BY SOYA BEAN OIL

By replacing butterfat with an emulsified soya bean oil and using a more unsaturated margarine in the diet, it was possible to reduce the serum cholesterol level in a series of patients.

A great deal of activity has occurred in recent years investigating the possible relationship between dietary fats and blood lipids with particular emphasis on cholesterol, and also the possible relationship between blood lipids and atherosclerosis.

Earlier issues of this journal have discussed the problem in great detail (*Nutrition Reviews* **15,** *1* (*1957*); **16,** *42* (*1958*); **17,** *291* (*1959*)). Since the dietary consumption of fats, particularly in the form of butter and milk, is high in Finland and also elevated serum cholesterol values and atherosclerosis are common, this problem may be of great practical significance in that country.

Recently, O. Turpeinen and associates (*Lancet* **I,** *196*, (*1960*)) studied the effect of replacing milk fat by emulsified soya bean oil in the diet of patients in two different mental institutions. The subjects were maintained on diets similar in calorie, protein and total fat content, except that patients in hospital "E," in place of butter or whole milk, were given margarine and skim milk supplemented with a 3.5 per cent emulsion of soya bean oil. The margarine used was a special type, considerably more unsaturated than the brands commonly on the market. Thus, although the patients in hospital "E" received a diet with a fat content similar to the control patients in hospital "C", the unsaturated fatty acid content of their diet was greatly increased and, conversely, the saturated fatty acid level decreased. The group in hospital "E" consisted of 117 patients with an average age of 49 years, and the control group in hospital "C" consisted of 53 patients with an average age of 48 years.

Before the start of the diet, the patients in hospital "E" had a mean serum cholesterol content of 235.7 mg. per cent, while after six months on the diet high in unsaturated fatty acids, the average blood cholesterol level had decreased to 215.3 mg. per cent. The difference between the two cholesterol levels was statistically significant ($P < 0.001$). During the same period, the average serum cholesterol value of the control patients in hospital "C" rose from an initial value of 252.7 mg. per cent to a final value of 262.7, although in this case the difference between the two serum cholesterol levels was not statistically significant. The decrease in the blood cholesterol in the patients maintained on the diet in which the milk fat was replaced by soya bean oil was greatest for those with the highest initial cholesterol level.

While there is no doubt that there was a statistically significant decrease in the serum cholesterol level of patients maintained for six months on a diet in which the only change was the replacement of certain animal fats by a more unsaturated vegetable fat, the study has not yet proceeded long enough to determine whether or not there is any influence on the frequency of coronary artery disease and other atherosclerotic manifestations along with the decrease in cholesterol level. It also should be noted that the dietary regimen employed by these investigators is not an unusual or intolerable one and is a type of feeding program that could be used on large populations without meeting too much opposition.

GLUCOSE METABOLISM IN THE RED BLOOD CELL

The normal human red blood cell metabolized 11 per cent of the glucose used by way of the aerobic phosphogluconic pathway and 89 per cent by way of the anaerobic Embden-Meyerhof scheme.

The erythrocyte is not a typical cell, since in the mature form those of mammals lack a nucleus. However, because it is easy to obtain in an uninjured form from all types of subjects and because of its keystone position in the transference of many substances, it becomes an important cell to study. Recently, J. R. Murphy (*J. Lab. Clin. Med.* **55,** *286* (*1960*)), in the second of a series of studies on erythrocyte metabolism, reported investigations concerning the utilization of glucose.

The red blood cell can metabolize glucose to carbon dioxide and lactic acid by two different pathways. One general scheme is the anaerobic Embden-Meyerhof route of glycolysis. In this pathway, glucose is eventually metabolized to lactic acid and there is a transfer of the energy of the glucose molecules to the high energy phosphate bonds of adenosinetriphosphate. No carbon dioxide is produced in this pathway. A second route of glucose metabolism involves the aerobic phosphogluconic pathway (glucose monophosphate shunt). In this pathway, carbon dioxide is formed from the first carbon of glucose (position 1) and the energy is made available in the form of reduced triphosphopyridine nucleotide. Since the tricarboxylic acid cycle does not function or is not present in the mature erythrocytes, the only known mechanism for the production of carbon dioxide from glucose in the red cell is by way of the phosphogluconic pathway.

Murphy studied blood obtained from healthy human subjects who had not donated more than 100 ml. of blood in the previous six months. This care in choosing donors was necessary in order to have a normal age distribution of the red cell in each sample. The blood was defibrinated using glass beads and then centrifuged twice. The buffy coat was removed after centrifugation, with only the loss of a minimum number of red cells. The cells were suspended in enough autologenous serum to produce a hematocrit of 50 ± 3 per cent. Under these conditions there were less than 50 white cells per cubic millimeter.

The amount of glucose utilized was considered to be the difference between that present in the blood before the incubation and the amount remaining after the study was terminated. The relative and absolute amount of glucose metabolized by the phosphogluconic pathway was calculated as the difference between the total amount of glucose metabolized and the amount of $C^{14}O_2$ recovered when the erythrocytes were incubated with glucose uniformly tagged with radioactive carbon, *i.e.*, with no predominance of the tag in any one of the six carbon atoms. This substance could be used as an index since the phosphogluconic pathway is the only route for the production of $C^{14}O_2$ from tagged glucose by the red cell.

It was found by this investigator that at pH 7.5 an average of 11.1 ± 0.6 per cent of the glucose metabolized in the normal red cell occurred by way of the phosphogluconic pathway. On studying the effect of pH on the rate of utilization of glucose, it was found that over a pH range of 6.8 to 7.8 the rate of utilization of glucose by the phosphogluconic pathway did not change appreciably. However, the total utilization of glucose fell as the pH decreased. Thus the total amount of glucose utilized was 45 per cent greater at pH 7.8 than at 7.5, and when the pH was 7.1 the rate of glucose utilization fell to about one half of that occurring at pH 7.5. Since the total utilization of glucose decreased as the pH decreased, with little change in the rate of that being metabolized by the phosphogluconic pathway, it appears that under more acidic conditions a larger share of the glucose is metabolized by the phosphogluconic pathway.

On the average, 11 per cent of the total amount of glucose metabolized in the red blood cell at pH 7.5 is by way of the aerobic phosphogluconic pathway, resulting in 25 per cent of the available energy of glucose being metabolized in the form of triphosphopyridine nucleotide. On the other hand, 89 per cent of the glucose used by the red blood cell is metabolized by way of the aerobic Embden-Meyerhof scheme, resulting in 75 per cent of the energy from glucose in the form of adenosinetriphosphate.

The tracing of the different metabolic pathways of glucose in the erythrocyte is an important contribution to the physiology of this unique and important cell. Also it opens possible avenues of investigation as to the mechanism of *in vivo* hemolysis which occurs with certain drug sensitivity reactions.

NUTRITIONAL SIGNIFICANCE OF THE RUMEN

Rumen fermentation causes metabolism of cellulose to acetic and butyric acids, hydrogenation of unsaturated fats, and combines degradation of protein with synthesis of essential amino acids.

The papers have now been published that were read at a symposium of the Nutrition Society in England on "Rumen Function". This has been a major field of fundamental research in British Agricultural Research Institutes for many years.

C. C. Balch (*Proc. Nutr. Soc.* **18,** *97 (1959)*) gave an introductory description of the physical structure of the ruminants' four stomachs, and of the observations made by cinematographic x-ray techniques on the movements of food ingesta during rumination. At the end of a meal the reticulo-rumen of a Shorthorn cow may contain as much as 200 pounds of food, and it rarely contains less than one half of this weight. Food particles, especially the larger ones may remain in the organ for several days and will only pass on to the omasum when they have become finely divided.

B. H. Howard (*Proc. Nutr. Soc.* **18,** *103 (1959)*) described studies made of the metabolism of food carbohydrates carried out with washed-cell suspensions of rumen bacteria. Cellulose, the most important of the polysaccharides undergoing breakdown in the rumen, is attacked mostly by bacteria with extremely strict anaerobic requirements. The main end products of their fermentation are acetic, lactic and succinic acids; but under the reducing conditions of the rumen these are further metabolized, largely to propionic and acetic acids. Fatty acids, in various proportions, seem also to be the end-products of xylan fermentation. Starch, which may be present in large amounts in rations fed to dairy cows, is also rapidly attacked by a variety of organisms present in the rumen, yielding a mixture of short-chain organic acids.

In addition, other organisms appear to synthesize and store as a reserve carbohydrate starch-like polysaccharides that give an iodine reaction. These are not, however, entirely built up from glucose units, and one example has been shown to consist mainly of galactose, rhamnose and uronic acid units.

Discussing the problem of protein utilization, M. J. Head (*Proc. Nutr. Soc.* **18,** *108 (1959)*) points out that the amino acid composition of the food is less important to the dairy cow than to single-stomached species. However, it is now known that, while the rumen microflora can and do synthesize essential amino acids for condensation into microbial protein, the composition of the rumen contents passing on for digestion and absorption in the true stomach and intestines can still vary with the type of protein originally fed.

One important factor influencing the fate of protein in the rumen is its solubility. With a high level of soluble material there is considerable microbial degradation of protein with the production of ammonia which diffuses into the blood stream. Much of this is converted into urea in the liver and excreted, but some returns to the rumen via the saliva and may be re-synthesized into protein if the necessary carbohydrate energy sources are also available. One consequence of this phenomenon is that proteins can be improved for ruminant feeding by such processes as heat treatment and hardening with formaldehyde, which would have the opposite effect if the proteins were intended for non-ruminants.

In a further paper, G. A. Garton (*Proc. Nutr. Soc.* **18,** *112 (1959)*) summarizes the changes undergone by food lipids during their period in the rumen. Of these, the most interesting is a partial hydrogenation of the unsaturated fatty acids in the glycerides present. Measurements of changes in iodine value, reported by several workers, have all indicated that more than one half of the

double bonds initially present were reduced. However, double bonds in a conjugated state appeared to be resistant to bacterial hydrogenation; this was the case for both fatty acids and carotene. There is no evidence for synthesis of the essential fatty acids in the rumen.

Dietary fat seems of little importance in the secretion of milk fat by dairy cows, but milk deficient in fat does result from the feeding of a diet low in roughage, or with roughage in a finely ground form (J. A. F. Rook, *Proc. Nutr. Soc.* **18,** *117 (1959)*). This is so even when a high level of energy continues to be fed in the form of easily digested starch. It is thought that butterfat synthesis normally occurs through acetic and butyric acids that become available from the fermentation of cellulose and other structural polysaccharides in the rumen. One would expect this fermentation to be reduced if either less roughage were fed, or if it were so finely divided that it stayed in the rumen for only a short period. Some, but not all, experiments have shown an improvement in butterfat levels after the injection of acetate or butyrate under these conditions.

Starch is, of course, also fermented in the rumen to short-chain acids, but propionic acid predominates and this acid does not appear to be used in fatty acid synthesis. On the contrary, it has a depressive effect.

Finally, A. T. Phillipson (*Proc. Nutr. Soc.* **18,** *131 (1959)*) considers the implications of rumen function for the remainder of the intestinal tract and for the animal as a whole. The special system evolved for the digestion of rough food in ruminant species demands a considerable turnover of the animal's resources. In a sheep, the daily secretion of saliva is of the order of ten liters, and of abomasal juice a further five liters. As the saliva is isotonic with plasma in its sodium content, and the quantity of sodium in most of the food eaten is small, a highly efficient system for the re-absorption of intestinal sodium is essential.

From various estimates, it appears that over 60 per cent of the dry matter in a ruminant's food is absorbed from the digestive tract while in the rumen and reticulum, *i.e.*, before the food has been in contact with the ordinary gastric and intestinal digestive enzymes. Further, estimates of the quantities of the short-chain fatty acids entering the blood stream of sheep and cattle indicate that these are, in each case, sufficient to satisfy at least the maintenance requirements for energy. The short-chain fatty acids are, of course, entirely the end-products of microbial metabolism.

Thus, concerning the nitrogenous components of the food, it is difficult to strike a balance between the losses caused by degradation of protein nitrogen to ammonia and the gains caused by the microbial synthesis of amino acids essential to the host animal.

So far, work in this field has largely been of a fundamental and exploratory nature. It can already be seen, however, that understanding of these phenomena will enable a more rational choice of feedstuffs, the processing of foods especially for ruminants, as well as feeding regimes adjusted according to the desired level of butterfat production.

ALTERATION OF YOLK FATTY ACIDS

Conditions have been defined by which the polyunsaturated fatty acid content of egg yolks can be varied within considerable ranges.

According to earlier results of R. Reiser (*J. Nutrition*, **40,** *429 (1950)*), the proportions of the various lipid fractions of egg yolk appear to be unaltered by the fat content of the diet. Reiser showed that the total lipid, phospholipid and cholesterol content was the same in hens fed either a fat-free diet or one containing 4 per cent of cottonseed oil.

However, from theoretical considerations, the fatty acid composition of egg yolk may well vary as a function of the diet. It is, therefore, not surprising that somewhat variable results have been obtained by different workers who have analyzed the fatty acid composition of egg yolk. The analyses of R. W. Riemenschneider *et al.* (*J. Biol. Chem.* **126,** *255* (*1938*)) differ from those reported by F. B. Shorland (*New Zealand J. Sci. & Technology* **33B,** *224* (*1951*)). Of course, analytical methods and genetic differences may account, in part, for such differences, but the egg yolks analyzed by Shorland were from hens of unknown dietary history, whereas those analyzed by Riemenschneider *et al.* were from hens that had been fed 2 per cent of cod liver oil and 7 per cent of fish meal (which may account for the relatively high level of C_{22} unsaturated fatty acids found).

E. M. Cruickshank (*Biochem. J.* **28,** *965* (*1934*)) and Reiser (*J. Nutrition* **44,** *159* (*1951*)) present evidence that the unsaturated fatty acid portion of the egg yolk is most susceptible to dietary manipulation. Cruickshank reported variations in fatty acids of egg yolks from hens fed high levels (28 per cent) of various fats. On feeding hens palm kernel oil (a highly saturated fat) she obtained only 16.1 per cent of the total fatty acids as linoleic acid, but on feeding a highly unsaturated fat (hemp oil) the linoleic content of the yolk was 41.7 per cent. Reiser showed that either a fat-free diet or the presence in the diet of bayberry tallow would result in reduction of the dienoic acid content of the yolk to very low levels. Even at low dietary fat levels (2 per cent) the polyunsaturated fatty acid fraction could be increased by feeding tung oil or codliver oil.

A study by A. S. Feigenbaum and H. Fisher (*Arch. Biochem. Biophys.* **79,** *302* (*1959*)) demonstrated that when various oils were fed to hens at 10 per cent of the diet the composition of the yolk fat was influenced only by the polyunsaturated fatty acids, whereas the composition of the body fat was influenced by either saturated or unsaturated fatty acids.

With the advent of gas-liquid chromatography, an improved analytical technique can be brought to bear on this problem. P. D. Wheeler, D. W. Peterson and G. D. Michaels (*J. Nutrition* **69,** *253* (*1959*)) have fed hens diets containing 5, 10, 15, 22.2 or 30 per cent of safflower oil; 22.2 or 30 per cent of linseed oil; and 10 per cent of cottonseed, corn or soybean oil. The total fatty acids of the yolk fat were determined by alkaline isomerization and by gas-liquid chromatography and their distribution compared with that of eggs produced by hens on a stock diet or on a low-fat diet.

The results of this study corroborated those of Reiser (*J. Nutrition*, **40,** *429* (*1950*)) in that the total lipid and free and esterified cholesterol content of the eggs from hens fed up to 30 per cent of oil did not differ from the values obtained with eggs from hens on the stock or control diet. However, in contrast to the findings of Reiser, the phospholipids were found to be considerably higher on the high-fat diets. Eggs produced by hens on a 20 per cent safflower oil diet averaged 1.8 g. heavier than those from hens on a stock diet. The flavor of the "safflower eggs" did not differ from that of control eggs, but eggs from hens on high linseed oil diets were reported by a taste panel to have off-flavors.

It was found that the linoleic acid content of eggs from hens fed safflower oil or cottonseed oil was approximately proportional to the level of this acid in the diet. However, in eggs from hens fed diets containing linolenic acid (including corn oil, soybean oil and linseed oil) linoleic acid was not deposited as efficiently as in those from hens fed safflower oil or cottonseed oil. The authors suggest an antagonistic or inhibitory effect of linolenic acid upon the incorporation of linoleic acid into yolk fat.

Analyses of eggs from hens on the linseed oil diet revealed that the increases in yolk levels of unsaturated fatty acids were ac-

companied by decreases in the monoenoic acids and palmitic acid but that the proportion of stearic acid remained the same. On all other diets, the principal changes accompanying increases in linoleic acid were decreases in oleic and palmitoleic acids. Only at about 40 per cent of linoleic acid was a slight decrease in palmitic acid observed and no change occurred in the proportion of stearic acid.

The levels of tetraenoic, pentaenoic and hexaenoic acids were low and variable in all diets. The authors could ascribe none of the changes to the diets used in these experiments.

It appears from the foregoing that conditions can now be defined which make it possible to vary the linoleic acid content of eggs. In the future, we can expect similar applications to a variety of grown foodstuffs.

INCAP ALL-VEGETABLE PROTEIN MIXTURES FOR HUMAN FEEDING

Biologic testing with rats and chicks has demonstrated the efficacy and safety of high-protein indigenous vegetable mixtures prior to trials with infants and children.

The manufacture of vegetable protein mixtures from low-cost ingredients available or potentially available in a given geographic region provides a practical answer to the problems of protein malnutrition, particularly in children, in many areas of the world. In planning such mixtures, at least eight factors must be considered (M. Béhar *et al.*, *Ann. N. Y. Acad. Sci.* **69,** *954 (1958)*): (1) the amino acid content of the individual ingredients and the final product; (2) the possible presence of toxic or interfering factors; (3) the need for obtaining exact specifications of each of the components; (4) the necessity of avoiding processes that damage the quality of the protein; (5) the desirability of using products of local origin; (6) the fact that the final product must be inexpensive and easily preserved; (7) the requirement that it be easily prepared in the home as an infant food by mothers of low-income families; and (8) the demand that it must not run counter to existing dietary habits and prejudices.

In addition to these requisites, the product should not be recommended for commercial production and mass consumption until four types of biologic trials have been carried out: (1) testing the product in at least two animal species; (2) demonstration of the biologic value of its protein by animal growth studies; (3) testing its acceptability and effectiveness in careful studies with children; and (4) field trials in selected communities or small population groups.

These principles and procedures have guided the workers at the Instituto de Nutricion de Centro America y Panama (INCAP) in developing a vegetable mixture, now entering commercial production, for the prevention of protein malnutrition in infants and young children in Central America. The history of the development of this all-vegetable protein mixture for human feeding has begun to be detailed in a new series of publications from INCAP (R. L. Squibb, M. K. Wyld, N. S. Scrimshaw and R. Bressani, *J. Nutrition* **69,** *343 (1959)*; Bressani, A. Aguirre and Scrimshaw, *Ibid.* **69,** *351 (1959)*).

The first paper describes in part the biological trials in rats and baby chicks of various food ingredients and combinations which preceded trials with children. Four independent trials were carried out with weanling white rats of the IAN (Instituto Agropecuario Nacional, Guatemala) Wistar strain colony. Three independent trials were carried out with three-day-old, straight-run baby New Hampshire chicks. The diets tested all contained approximately 25 per cent of crude protein (the protein level desired in a product for infant and child feeding) and were composed of crude sesame oil meal or refined

sesame flour, lime-treated corn or ground raw corn, cottonseed flour or dried skim milk, kikuyu leaf meal (as a source of vitamin A), *Torula* yeast or a B-complex vitamin mixture, vitamin D or cod liver oil, and (in certain trials) L-lysine, DL-valine, arginine, gelatin and corn cob meal.

During trial one of the rat-feeding experiments, a comparison was made between crude sesame oil meal and refined sesame flour for a nine-week period with and without the addition of skim milk. Data analysis revealed no significant differences in the weights of the rats fed the two sesame products, with or without added skim milk. Refined sesame flour without skim milk, however, resulted in an efficiency of feed utilization (gram feed per gram gain) superior to that with crude sesame oil meal, but when skim milk was added to both types of sesame there was no difference in efficiency.

During rat trial two, cottonseed flour (9 per cent) and two levels of dried skim milk (9 and 14.3 per cent) were tested as supplements to the sesame flour for a period of eight weeks. The supplements tested were substituted for a part of the protein of the sesame flour. Rats receiving the higher skim milk supplement gained slightly more and showed a better feed efficiency, but the differences in growth were not statistically significant.

During rat trial three, cottonseed flour and dried skim milk, with and without L-lysine, were tested as supplements to sesame flour in the all-vegetable mixture for a period of eight weeks. As before, the supplements were substituted for a part of the protein of the sesame flour. The data showed no significant differences in the growth of the rats among the experimental and control groups. However, in each instance, the addition of L-lysine improved the efficiency of feed utilization. The authors attributed the lack of significant growth stimulation by lysine to the high protein content (25 per cent) of the diets and to the fact that the experiment was continued beyond the usual four-week period of rapid growth response.

During rat trial four, the effects were tested of supplements of L-lysine and DL-valine (the amino acids most deficient in the all-vegetable mixture when compared to milk) in diets containing 15 per cent protein derived mainly from sesame and cottonseed flour. A depression of growth rate and feed efficiency, not significant at the 5 per cent level, was noted on addition of DL-valine. A significant increase in growth rate and feed efficiency was observed on addition of L-lysine.

During trial one of the chick-feeding experiments, the effect of lime treatment of corn on chick growth and feed efficiency was observed by comparing lime-treated and raw corn (with and without supplementation by B-complex vitamins) as components of the all-vegetable mixture containing sesame and cottonseed flours. Raw corn produced significantly greater growth and efficiency of feed utilization whether or not B-complex vitamins were added. The greater growth occasioned by the addition of B-complex vitamins was not statistically significant.

During chick trial two, the effects of adding gelatin, arginine and lysine to a sesame flour, cottonseed flour, lime-treated or raw corn combination were measured. Gelatin was tested because it is a good source of arginine for the chick and because of the possibility that the inferior growth obtained with lime-treated corn might be due to the loss of 18 per cent of arginine occurring as a result of lime treatment (Bressani and Scrimshaw, *J. Agr. Food Chem.* **6**, 774 (*1958*)). Addition of gelatin decreased growth and feed efficiency, but the addition of arginine increased both, none of the differences, however, reaching the 5 per cent level of significance. Addition of L-lysine, however, singly or in combination with the other supplements tested, significantly increased growth and feed efficiency of the lime-treated corn diets.

Since chick trials one and two had indicated that the sesame flour, cottonseed flour, lime-treated corn combination was lysine de-

ficient for chicks and that the use of lime-treated corn resulted in lower growth rates in chicks, an attempt was made during chick trial three to improve digestion and absorption through the purely mechanical effect of giving three levels of corn cob meal in place of part of the lime-treated corn to three of eight groups of chicks. In another three of the eight groups tested, the supplemental value of L-lysine was tested at 0.2, 0.3 and 0.4 per cent of the diet. Lime-treated and raw corn were compared directly in the remaining two groups.

Lime-treated corn again produced lower growth. Addition of corn cob meal to lime-treated corn diets improved chick growth to values comparable to those obtained with raw corn. L-lysine addition produced greater growth and feed efficiency at all levels, but had its greatest effect on growth at the 0.4 per cent level. The authors interpreted these results to indicate that the physical characteristics of the raw corn diets, rather than any direct nutritional effects, were responsible for their superiority for chicks over the lime-treated corn diets.

These experiments and other previously collected laboratory data led to the adoption of a final formula for human trials. This formula, designated INCAP Vegetable Mixture 8, contained in per cent: dry corn masa, 50 (masa is obtained by cooking corn in a lime solution and subsequently drying and grinding it); sesame flour, 35; cottonseed flour, 9; kikuyu leaf meal, 3; and *Torula* yeast, 3. The mixture contained adequate vitamins and minerals (Béhar *et al., Ann. N. Y. Acad. Sci.* **69,** *954 (1958)*). Comparison of the essential amino acid pattern of this formula with the pattern of cow's milk (M. L. Orr and B. K. Watt, *Home Economics Research Report No. 4, U. S. D. A., Washington (1957)*) and the FAO Reference Protein (*Food and Agriculture Organization Nutritional Studies No. 16, p. 24 (1955)*) suggests that the INCAP mixture is limiting in lysine, that tryptophan is the second most limiting amino acid and that methionine (methionine plus cystine) is the third. The protein score of the mixture is about 67 per cent, indicating that the mixture is within the lower range of protein of animal origin.

The biologic trials described satisfied the authors as to the lack of toxicity of any of the ingredients or combinations of ingredients in the mixture and encouraged the probability that the mixture would prove adequate for the nutritional needs of growing infants and children. Subsequent experience in infants and young children, to be detailed later in this series confirms the adequacy of the rat and chick testing methods in evaluating vegetable mixtures.

The second paper in this series (Bressani, Aguirre and Scrimshaw, *J. Nutrition* **69,** *351 (1959)*) describes experiences of INCAP workers in feeding chicks Vegetable Mixture 8 in which the lime-treated corn of the original mixture was replaced by raw yellow corn, grain sorghum (one species of a genus of tropical cereal grasses), rice or buckwheat. Each cereal grain tested was substituted completely for 50 per cent of the corn masa flour of the basal diet. The complete diets were diluted to 75 or 80 per cent of their original value to give protein concentrations of approximately 21 or 22 per cent.

Buckwheat resulted in better growth, feed efficiency and protein efficiency (gram of weight gained per gram of protein consumed) than any of the other cereal grains substituted for part of the corn. Raw yellow corn and rice produced slightly greater growth than sorghum and the basal INCAP Vegetable Mixture 8. Feed efficiency values for the basal diet, however, were somewhat better than those obtained when yellow corn and rice were utilized. Feed efficiency and protein efficiency values of the rations containing sorghum were the lowest. Protein efficiency values of the basal diet, yellow corn and rice were essentially equal. Lysine supplementation at the 0.4 per cent level improved the growth, feed efficiency and protein efficiency of all the experimental rations, being most effective during rice substitution.

The results of these experiments suggest that any of the four cereals tested could be substituted for all or part of the corn masa flour in INCAP Vegetable Mixture 8 if economic and agricultural factors make this desirable. They also point up interesting problems for future investigation, particularly whether the differences observed among the variously substituted mixtures are due to differences in amino acid availability, a less favorable amino acid pattern or lower digestibility.

Preliminary reports have been published (Scrimshaw *et al., Amino Acid Malnutrition, XIII, p. 28. Annual Protein Conference, Rutgers University Press (1957)*; Béhar *et al., Ann. N. Y. Acad. Sci.* **69,** *954 (1958)*) which indicate good results from the feeding of INCAP Vegetable Mixture 8 to young children. More extensive clinical experience, to be detailed in the next paper in this series, bears out the impressions expressed in the preliminary reports. The development of "Incaparina" should serve as a model for similar work elsewhere as well as a warning that long years of carefully planned and deliberately executed research are requisite to obtaining an effective and thoroughly safe product for human consumption.

PLASMA AND BONE MAGNESIUM

A relationship between bone-ash magnesium and plasma magnesium has been demonstrated. However, the bone plays little or no part in the control of plasma magnesium in magnesium excess, but serves as a reservoir in deficiency.

It was shown by E. R. Orent, H. D. Kruse and E. V. McCollum (*J. Biol. Chem.* **106,** *573 (1934)*) that bone magnesium is depleted in rats kept on magnesium-deficient diets. The same was subsequently shown to be true for calves (C. E. Knoop, W. E. Krauss and C. C. Hayden, *J. Dairy Sci.* **22,** *283 (1939)*; W. H. Parr, *Vet. Rec.* **69,** *71 (1957)*), but in neither of these animals are the soft tissues appreciably depleted even under the most severe conditions of magnesium deficiency.

It thus appears that bone magnesium represents a store of this element that can be called upon under conditions of deficiency to supply the needs of the soft tissues. J. Duckworth and W. Godden (*Biochem. J.* **35,** *816 (1941)*) have shown, by killing rats at different times after introducing a magnesium-deficient diet, that bone magnesium can be mobilized rapidly. K. L. Blaxter (*Ciba Foundation Symposium on Bone Structure and Metabolism, p. 117. Churchill, London (1956)*) has related bone magnesium to plasma magnesium in calves slaughtered with differing degrees of hypomagnesemia developed as a result of protracted feeding of milk.

During a study by R. H. Smith (*Biochem. J.* **71,** *609 (1959)*) on the development of hypomagnesemia in milk-fed calves, the opportunity was presented for a very detailed analysis of the relationship between such hypomagnesemia and changes in bone composition. Bones examined were the caudal vertebrae (which could be taken from the living animal), rib shaft, femur head and shaft and the first phalanges. At least ten successive samples could be obtained from one calf if sections containing only one vertebra were removed at a time.

The bone compositions in several calves fed normally and in two groups of calves developing hypomagnesemia were examined. The first group of calves developing hypomagnesemia consisted of animals fed almost exclusively on cow's milk supplemented with vitamin D, iron, copper and manganese. Caudal vertebrae were removed at approximately monthly intervals. The second group consisted of calves which, at about two to four weeks of age, were transferred

from a magnesium adequate diet to one of low-magnesium synthetic milk. Caudal vertebrae were removed before and after the change in diet. Preliminary experiments showed that compositions of most of the vertebrae in an individual animal were similar, so that one vertebra could be used to assess the composition of the remainder.

Although the ash contents of the different types of bones in the animal were somewhat variable, the only marked consistent difference was in the femur, which contained 20 to 30 per cent more ash than did the caudal vertebrae in both the normal and magnesium-depleted calves. The calcium in the ash was closely similar for the different bones in all the calves, as was the magnesium in the ash for the normal calves. In the magnesium-depleted calves there were marked differences among the ashes of the various bones. Of those studied, the femur shafts were depleted to the least extent and the vertebrae and first phalanges to the greatest extent. The author points out that the caudal vertebrae, therefore, offer a sensitive guide to the magnesium status of the skeleton, but that changes observed in these vertebrae during magnesium depletion do not exactly reflect the changes occurring in the skeleton as a whole.

There appeared to be a broad direct relationship between the rate of onset of hypomagnesemia in the different calves and the rate of depletion of their bone magnesium. Thus, drops in concentrations of magnesium in the plasma to 1.6 and 0.7 mg. per 100 ml. were associated with bone-ash magnesium figures of 0.60 to 0.67 and 0.40 to 0.48 per cent, respectively, whatever the rate at which the concentration of plasma magnesium fell. The plasma magnesium decreased only very slowly after reaching a level of about 0.7 mg. per 100 ml., and further depletion of bone magnesium occurred with relatively small decreases in the plasma concentration. When the last bone samples were taken (some animals died, presumably from magnesium deficiency) the mean plasma magnesium level was 0.47 mg. per 100 ml. and the mean magnesium in the bone ash was 0.27 per cent (about 38 per cent of normal).

Following an abrupt change to a low-magnesium diet, the depletion of bone magnesium appeared to occur at an approximately constant rate (presumably at a rate necessary to provide adequate magnesium for the growing soft tissue and for endogenous fecal excretion). Urine excretion of magnesium fell to nearly zero as soon as the calves were transferred to a magnesium-deficient diet. Plasma magnesium fell, rapidly at first and then more slowly. The growth rates of the calves and their consequent needs for magnesium were depressed.

When the bone-ash magnesium values of two of the calves fed on low-magnesium synthetic milk had fallen, respectively, to 84 and 68 per cent of the normal value, they were given daily injections of one gram of magnesium (sulfate). After five days of injections, the plasma magnesium of both calves ranged between 3.7 and 2.2 mg. per 100 ml. (about 160 and 95 per cent of normal). Following nine days of injections in one case and 16 days in the other, the bone-ash magnesium for both calves was found to be normal. Bone samples (vertebrae) taken periodically from each of the calves did not show any marked changes in ash content nor in the percentage of calcium in the ash concomitant with magnesium depletion.

The author points out that the above results suggest that magnesium behaves similarly to calcium in its lack of uniform depletion of different parts of the skeleton during deficiency. Calcium has also been shown to be removed more readily from cancellus bone than from compact bone in calcium-deficient sheep (D. Benzie *et al.*, *J. Agr. Sci.* **46**, *425* (*1955*)). The relationship between bone and plasma magnesium in calves slaughtered with varying degrees of hypomagnesemia is similar in general to that reported by K. L. Blaxter (*loc. cit.*).

Such small differences that exist may be partially accounted for by the fact that Blaxter examined bone from the metatarsal shaft whereas Smith used caudal vertebrae.

The results obtained suggest further that an equilibrium exists between bone and plasma magnesium and that normal bone, from a physiological standpoint, is virtually saturated with magnesium. This was especially indicated by the fact that daily injection of magnesium, so that the plasma magnesium did not fall appreciably below normal (and for a part of the time was considerably above normal), did not lead to any appreciable increase in bone magnesium above normal amounts.

Thus the bone plays little or no part in regulating concentration of plasma magnesium. Instead this control seems to be largely a function of changes in urine excretion. The excretion is high when the diet contains more than adequate magnesium (Smith, *Biochem. J.* **67,** *472 (1957)*; **70,** *201 (1958)*) and increases enormously when enough magnesium is injected so that plasma magnesium rises above normal (Smith, *Ibid.* **71,** *306 (1959)*). However, it decreases, eventually nearly to zero, when the magnesium available from the diet is decreased. Only when the available magnesium is so low as to be inadequate even with almost no loss in the urine, is bone magnesium apparently liberated.

M. J. Dallemagne and C. Fabry (*Ciba Foundation Symposium on Bone Structure and Metabolism, p. 14. Churchill, London (1956)*) have suggested that about two-thirds of bone magnesium is adsorbed on the surface of the bone-salt crystals, with the remaining one-third replacing calcium in the phosphate molecule. The author suggests that the adsorbed fraction may be, in fact, that portion which is available in magnesium depletion.

ABSORPTION OF CYSTINE AND CYSTEINE

The absorption of isomers of cystine and cysteine from the small intestine of the rat has been studied with a lumen-perfusion (in situ) technique and with an everted-sac technique.

Many worthwhile studies have been made of the efficiency of specific proteins in promoting growth, maintaining the nitrogen balance and supporting good health. However, for a basic understanding of the mechanism involved, it is necessary to know where and at what rates the various amino acids are absorbed from the intestine.

Although numerous investigations have been made in the past few years of the absorption of amino acids from the small intestine, cystine and cysteine have not been included in the compounds studied. This is due in part to the insolubility of cystine and the rapid oxidation of cysteine in oxygenated solutions. Recently, M. W. Neil (*Biochem. J.* **71,** *118 (1959)*) has employed a lumen-perfusion technique *in situ* which permits observation of absorption of amino acids in a defined segment under purely physiological conditions. The everted-sac technique was also used by Neil in some studies of cystine absorption.

Male albino rats weighing 200 to 300 g. were used, and dissection, intestinal cannulation and perfusion were carried out by conventional techniques. The perfusion pressure measured at the proximal end of a perfused segment was kept at 9 to 11 cm. water throughout each experiment with a total volume of the fluid (bicarbonate saline) amounting to about 55 ml. One ml. samples were removed periodically for analysis. Added cystine plus cysteine were determined polarographically with Tinsley polarographic equipment and then cystine

alone was measured after allowing the cysteine to react with iodoacetate.

In perfused segments of duodenum, jejunum and ileum, the potassium content of the whole tissue remained practically normal. The water content of the segments was only about 3 per cent higher than that of control samples. Apart from this slight edema, the mucosal epithelium was practically normal in sections from perfused segments. The well established inhibition of active absorption of glucose by phlorizin in such preparations was used as a functional test. Inhibition was unequivocally demonstrated.

Absorption of cystine and cysteine was studied by following the decrease of the amino acid in the perfusion fluid. The big advantage (as far as cysteine is concerned) of the preparation *in situ* is that it is possible to keep the perfusion fluid oxygen-free as the gut itself is oxygenated by the flow of blood.

In the jejunum the rate of absorption of L-cystine was found to be about 70 per cent of that of L-cysteine. Substitution of nitrogen for oxygen in the cystine absorption experiments had no effect on the rate of absorption of this amino acid. The rates of absorption of cystine clearly decreased in the order: duodenum, jejunum and ileum. The absorption of L-cysteine was also slower from the jejunum than from the duodenum.

The D-isomers of both cystine and cysteine were absorbed from rat duodenum at much lower rates than their L-isomers. These results provided evidence that an active process was involved in the absorption mechanism for the L-isomers in each case.

Since certain preliminary experiments indicated that the physiological state of the rat small intestine was reasonably well preserved with the everted-sac technique, the author decided to use this preparation for further studies on the nature of the transport of cystine across the rat intestinal wall.

Everted sacs of rat small intestine were prepared and incubated in bicarbonate saline, containing cysteine. The initial cystine serosal to mucosal concentration ratio was 1:0. After one hour the ratio was again determined. It was found that L-cystine was transported from the mucosal side to the serosol side of the sacs against this concentration gradient. No such transport of D-cystine occurred.

Since dinitrophenol inhibits phosphorylating processes that might be involved in active transport, the effect of this substance was ascertained with both the lumen-perfusion techniques and the everted-sac techniques. In the first instance, the presence of 0.3 millimolar 2,4-dinitrophenol had no influence on the absorption of D-glucose, L-cystine and L-methionine and did not alter the potassium and water contents of the perfused segments. However, in the everted-sac technique, such a large loss of potassium and increase in water occurred that the test for inhibition of cystine absorption was ruled invalid.

The author emphasizes the importance of comparing the results of absorption studies on the basis of the particular technique used (this should also be kept in mind when extrapolating for nutritional applications). For example, in the present case, the absorption routes of the metabolites were different in the two preparations. In the technique *in situ* the route approximated the normal, but in the everted-sac technique the amino acids had to traverse the entire intestinal wall. However, the conditions were more reproducible in the technique *in vitro* than in the technique *in situ* because the latter was subject to such factors as variation in the blood flow perfusing the gut capillaries, amounts of anesthetic required, degree of shock and degree of distention of the segment during the perfusion.

Unfortunately, from the standpoint of physiological nutritionists, the author presents no evidence citing the relative absorption of glucose or L-methionine as compared to L-cystine and L-cysteine.

LEAF PROTEIN AS HUMAN FOOD

A simple method of extracting protein from plants has been devised and the resulting protein product, while deeply colored with chlorophyll, is odorless, tasteless and suitable for protein supplementation in man.

In spite of the millions of hungry people in the world today, world population is increasing by about 40 to 50 million persons a year. When modern medicine and sanitation become more effective in the undeveloped regions this rate will undoubtedly increase, and with this there will be an increasing demand for all nutrients. Although the employment of improved methods of agricultural technology, increased irrigation and drainage, selection of crops to produce a greater yield per acre, and the eradication of plant disease and pests will lead to greater production of foodstuffs, there is doubt whether such methods can meet the increased demand for food. Protein will probably be in shortest supply since the foodstuffs that are easiest to produce, yield large amounts of calories but supply inadequate amounts of protein. Moreover, when foodstuffs are limited in supply the use of animals for converting plant protein into animal protein is too inefficient. Hence, in large areas of the world animal protein is used as a condiment rather than a source of utilizable nitrogen.

Recently, N. W. Pirie (*Lancet* **II**, *961 (1959)*) has discussed the possibility of converting leaf proteins into foodstuffs suitable for human consumption. It is logical to use such products since all of the proteins derived from animals have been produced by the animal organism directly or indirectly from plants. Thus a device has been made which simply and efficiently extracts proteins from plant products.

Fresh green leaves are first pulped and the juice expressed from the solid material. After straining the juice, the protein is coagulated by heating to 80°C and filtered off. The protein is then resuspended in water and filtered again. If the original leaf were strongly flavored this process can be repeated. The protein cake resulting from this procedure, while dark green in color due to the chlorophyll present, has little or no taste or smell and has the consistency of cheese or yeast. It contains 60 per cent water. At room temperature it will keep a week or so but slowly molds. Drying in air or in an oven yields a hard granular product, but it is possible to avoid this undesired consistency by either freeze-drying or drying it in the presence of flour or other materials. The plant proteins will keep indefinitely in the frozen state and can also be canned for storage.

One of the advantages of these protein products is that they are a mixture of a number of different plant proteins and, therefore, probably contain a better spectrum of amino acids. Thus the amino acid deficiency which may be present in any single plant protein tends to be modified by the presence of other proteins. Pirie believes that these proteins will be a valuable food even as the main protein of the diet, although not as good nutritionally as the standard animal proteins, casein or egg albumin. When nutritional studies were carried out on pigs it was found that the leaf proteins were as good or a little better than fish meal. Pirie also indicates that comparable findings (not yet published) were noted for chickens and rats.

Now that it has been shown that proteins fit for human consumption can be readily formed from many types of plant products, the question arises as what could best be used as raw material in areas where the need for proteins is most acute. The author suggests that there are, in general, three main types of leaves which could be used effectively. The first are wild leaves, but since these normally grow in areas unsuitable for large scale cultivation, collection

would be difficult. However, water plants such as papyrus and the water hyacinth (which in many cases are clogging waterways) would be easy to collect and would supply a ready source of protein. In addition, this process would help clear rivers and bays. The second type are the waste leaves, such as sugar beet tops in the temperate zones, while in the warmer regions the leaves of such crops as sweet potatoes, sugar cane, banana and cassava might be good sources of leaf protein. Technical difficulties are foreseen with some of these products, but these may be solved as they arise.

Finally, it would be possible to plant special crops to be harvested mainly for leaf proteins. There are many plants which grow luxuriously but, because they do not produce large seeds or tubulars or the leaves are unpalatable to livestock, cannot be used at present for food. Also, by raising special crops to obtain leaf proteins it might be possible in many cases to harvest the plants while young and less vulnerable to some pests and diseases which attack plants in the more mature state.

Obviously some of the difficulties which may arise in using these plant proteins on a large scale are food habits and preferences, particularly since the material is intensely green in color. This alone may prevent its widespread use if there is insufficient education. Also if 20 g. or more of such a protein is injected, part of the chlorophyll escapes and appears in the feces. The colored stool does not indicate that the protein is not being digested but rather that the chlorophyll is not being destroyed.

This simple device for extracting protein from plant leaves opens new areas in providing nutrition for a large population. Also the possibility exists that increased utilization of other parts of plants, presently harvested for only seeds or fruit, might be used to increase the protein supply of an area.

PHOSPHATIDE-INDUCED HYPERCHOLESTEREMIA

The results of experiments on laboratory animals suggest that intravenous infusions of phospholipids may cause mobilization of tissue cholesterol.

Infusions of various types of phospholipids have been shown to increase the concentration of cholesterol in the plasma of rats and rabbits (M. Friedman and S. O. Byers, *Proc. Soc. Exp. Biol. Med.* **94,** *452 (1957)*; *Am. J. Physiol.* **186,** *13 (1956)*). Hypercholesteremia develops when a solution of phosphatides from liver, brain, soybeans or yeast is infused over a six to 24-hour period (Byers and Friedman, *Am. J. Physiol.* **193,** *435 (1958)*). In an attempt to elucidate the mechanism of this phosphatide-induced hypercholesteremia, Friedman and Byers (*Ibid.* **195,** *185 (1958)*) studied the effects of infusions of lecithin on the cholesterol content of various organs of the rat.

Rats were given intravenously, without anesthetic, 3 ml. of a colloidal suspension of lecithin (3 per cent in a solution containing 5 per cent of dextrose) at the beginning of the experimental period. Thereafter they were infused continuously at a rate of 0.5 ml. per hour for 12- or 24-hour periods. Cholesterol concentration was determined by the method of A. Saifer and O. F. Kammerer (*J. Biol. Chem.* **164,** *657 (1946)*), in whole blood, plasma and tissues. Blood was obtained by bleeding the animals from the tail.

When a laboratory chow diet to which 3 per cent of cholesterol and 4 per cent of cottonseed oil had been added was pre-fed, the infusion of lecithin brought about a significant reduction in the concentration of cholesterol in the liver within 12 hours. However, when a sterol-free diet was fed,

the liver cholesterol concentration of rats infused for 24 hours with lecithin was higher than that of rats infused with dextrose alone or of rats not subjected to infusion. The infusion of dextrose alone resulted in some lowering of liver cholesterol concentration.

Twenty-four-hour infusion of lecithin resulted in a 75 per cent decrease in the cholesterol content of the adrenals of rats pre-fed a sterol-free diet. However, infusion of dextrose alone reduced the adrenal cholesterol concentrations from 121 mg. per g. to 54 mg. per g., a 55 per cent decrease. In rats pre-fed the cholesterol-enriched diet, a 12-hour infusion with lecithin resulted in a cholesterol content of the adrenals of 32 mg. per g. while infusion with dextrose alone gave a value of 55 mg. per g. Unfortunately no values were reported for control rats pre-fed the cholesterol-enriched diet but given no infusion. Infusion of lethicin or dextrose alone into rats fed either the sterol-free diet or the cholesterol-enriched diet caused no change in cholesterol concentration of the intestines, skin, lung and other viscera studied.

Analyses of whole blood following infusion showed an increase in cholesterol concentration of the same magnitude as that occurring in plasma, indicating that the excess plasma cholesterol did not originate in the formed elements of the blood. Also, the possibility that cholesterol or other sterols present in the phospholipid which was infused might account for the hypercholesteremia must be discounted because especially purified samples of phospholipid, essentially free of Lieberman-Burchard positive sterols, were as effective in producing hypercholesteremia as samples that were not highly purified.

In the interpretation of these results, the authors conclude that the infusion of phosphatides can reduce the cholesterol content of organs having a high concentration of cholesterol, such as the adrenals and the liver, the latter only in animals pre-fed a cholesterol-rich diet. They believe that the infused phospholipid has a "solubilizing or chemotaxic" effect on cholesterol in various tissues including aortic atherosclerotic plaques (Friedman, Byers and R. H. Rosenman, *Proc. Soc. Exp. Biol. Med.* **95,** *586 (1957)*), thus transferring tissue cholesterol to the blood. However, the amount of cholesterol removed from the various organs studied was not sufficient to account for the amount of cholesterol found in the plasma after phospholipid infusion. The authors suggest that the depletion of 10 to 21 mg. of cholesterol (the amount required to account for the observed hypercholesteremia) from the total body pool of 300 to 400 mg. could not be detected by the quantitative determination used.

They discount an effect of infused phospholipid on the metabolism or mobilization of cholesterol in the liver because the infusion of phosphatide resulted in even higher plasma cholesterol levels in hepatectomized rats than in normal animals (Byers and Friedman, *Proc. Soc. Exp. Biol. Med.* **92,** *459 (1956)*). The plasma cholesterol concentrations were higher in hepatectomized rats presumably because less of the phospholipid was removed from the blood in the absence of the liver and the resulting higher plasma phospholipid concentration caused more severe hypercholesteremia.

Since prefeeding a cholesterol-rich diet enhances the hypercholesteremia of phosphatide infusion (Friedman and Byers, *Am. J. Physiol.* **192,** *546 (1958)*) and since dietary cholesterol depresses cholesterol synthesis, the authors have taken this as additional evidence against the possibility that increased cholesterol synthesis is responsible for the phosphatide-induced hypercholestermia.

The hypothesis of Friedman and Byers concerning the origin of excess plasma cholesterol in phosphatide-induced hypercholesteremia appears reasonable, but the possibility that changes in cholesterol metabolism occur secondarily to hypercholesteremia has

not been eliminated. A small increase in synthesis in several or all organs from which cholesterol is removed by the lipemic serum could restore the original tissue concentration and account for the fact that little change is observed in the over-all tissue concentrations of cholesterol when the serum level rises. However, direct studies of the effects of infusion of phospholipids on the synthesis and catabolism of cholesterol are needed to appraise this possibility.

In evaluating the conclusions, the possible effects of the stress to which the animals were subjected must also be considered, particularly in view of the reduction in liver and adrenal cholesterol demonstrated upon infusion of dextrose alone; however, infusion of dextrose alone produced no hypercholesteremia.

These observations on the effects of phospholipid infusions on serum and tissue cholesterol concentrations appear to have received little attention. They should be investigated in more detail, particularly in view of the possibility that the infusion of phospholipid may lead to the removal of cholesterol from atherosclerotic plaques.

RIBOFLAVIN AND ADRENAL CORTICAL METABOLISM

Rats fed a diet deficient in riboflavin developed signs compatible with ariboflavinosis, i.e., impairment of adrenal ascorbic acid depletion following stress. However, pair-fed control animals showed a similar response.

Although riboflavin was found by P. György to be necessary for growth of rats (*Nutrition Reviews* **12,** *97 (1954)*), the details of its mode of action were unknown. One of its functions seemed to relate to the adrenal cortex. Animals deficient in riboflavin were found to have lost their gluconeogenic response to anoxia (B. R. Forker and A. F. Morgan, *J. Biol. Chem.* **209,** *303 (1954)*; **217,** *659 (1955)*; *Nutrition Reviews* **13,** *19 (1955)*), but this could be restored by administration of riboflavin or of cortisone. Another report described the inability of deficient animals to show diuresis after being loaded with water (*Nutrition Reviews* **14,** *120 (1956)*). This defect also was corrected by cortisone or corticotrophin.

These considerations led G. G. Slater (*Endocrinology* **65,** *731 (1959)*) to study further the role of riboflavin in the metabolism of the adrenal cortex in animals, as well as the effect of total dietary restriction on this function. He chose rats weighing 50 to 200 g. and fed them (1) a high-carbohydrate, riboflavin-free diet, or (2) a high-protein, riboflavin-free diet. Galactoflavin, a metabolic antagonist to riboflavin, was added to the ration of some animals in the amount of 1 mg. per gram of diet. Others were given riboflavin in their ration. Stress was applied by surgically removing the left adrenal gland and, one hour later, the right adrenal. The ascorbic acid content of each adrenal was then determined.

In the normal animal, this stress significantly reduced the content of adrenal ascorbic acid. In the present study, loss of this response was considered evidence of riboflavin deficiency. In young animals (weighing 80 g.) the response was lost after ten days of a diet high in carbohydrate and containing galactoflavin. In older animals the response became impaired after three to five weeks.

Severity of the stress seemed to be a factor, and excessively severe surgical manipulation was capable of evoking depletion of adrenal ascorbic acid in a group of animals which did not respond to the milder operative procedure.

Dietary intake also was important. When control groups of animals were allowed only enough food to maintain their weight within a range similar to that of the deficient

animals, this restriction of their total diet checked the depletion of adrenal ascorbic acid.

Intraperitoneal administration of 1.5 mg. of riboflavin to deficient animals failed to restore their response to stress applied three hours later. It was also significant that pair-fed controls (one group deficient and the other supplemented with riboflavin) displayed a similar degree of impairment of adrenal ascorbic acid depletion following stress.

Forker and Morgan (*loc. cit.*) had earlier demonstrated that depletion of adrenal ascorbic acid failed to occur in riboflavin-deficient rats subjected to stress, whereas pair-weighed but riboflavin-supplemented animals showed a significant response. Slater confirmed their findings on riboflavin-deficient rats but suggested that, since the deficient animals ate so poorly, other nutritional deficiencies might account for the abnormality. This suspicion was justified by the fact that his pair-fed control animals receiving 0.1 mg. riboflavin per gram of diet developed the same impaired response to stress as the riboflavin-deficient animals, in disagreement with the findings of Slater and Morgan.

Certain differences, however, between the two sets of experiments might account for this discrepancy. Forker and Morgan applied a more drastic form of stress, namely anoxia, and the animals were given a deficient diet for 11 weeks and starved for 16 hours prior to testing. Slater's group of animals were fed a similar diet, but the stress of unilateral adrenalectomy was less drastic and the animals were not in a fasting state. The inclusion of a group of animals fed a high-protein diet appears to eliminate a deficiency of amino acids as a limiting factor in the synthesis of either corticotrophin or adrenal cortical hormones. However, it seems apparent that the limitation of total diet did impair the pituitary-adrenal axis sufficiently to produce a measurable change compatible with a simple effect of starvation or with a deficiency of an unidentified factor.

The fact that corticotrophin was capable of depleting the adrenal gland of ascorbic acid, even though the animals were deficient in riboflavin, would tend to indicate impairment of release of the pituitary hormone. However, severe stress such as extensive laparotomy resulted in significant depletion of adrenal ascorbic acid. Slater considers the possibility that severe stress resulted in release of epinephrine or some other humoral agent, although he considers it more likely that the hypothalamic-pituitary system was activated to release corticotrophin.

This report emphasizes the difficulty of assessing a single physiologic response to depletion of an essential factor from the diet. It further emphasizes the need for meticulous control in nutritional studies and for the inclusion of pair-fed animals in any experiment which may alter the appetite of the subjects.

NOTES

Mannose Toxicity in the Honeybee

Mannose, a well utilized sugar by mammalian species, is metabolized first by being converted to the mannose-6-phosphate using adenosine triphosphate as a phosphorus donor. This is then followed by a second reaction which converts mannose-6-phosphate into fructose-6-phosphate, a common intermediate for glucose, fructose and mannose metabolism. The initial reaction of phosphorylation is catalyzed by hexokinase, an enzyme which performs this reaction for glucose, mannose and fructose. The second reaction is catalized by phosphomannose-isomerase.

Many years ago it was reported that the sugar mannose was very toxic for honeybees. Recently this problem was reinvestigated by A. Sols, E. Cadenas and F. Alvarado (*Science* **131,** *297 (1960)*). These investigators studied three groups of 20 worker honeybees, allowing each group to feed on one of the following liquids: molar mannose, molar glucose solutions or water. Those fed the mannose had a 50 per cent mortality within one and one-half hours and over 90 per cent were dead in three hours. Of the controls receiving water, 90 per cent were dead within 12 hours, while 90 per cent of those fed the glucose solution were still surviving within three hours after the feeding period.

These investigators determined the hexokinase and isomerase activity in homogenates of whole bees killed by freezing at −20°C after a one-hour fast. It was found that the homogenates of the honeybees had a high activity for the phosphorylation of glucose, mannose and fructose and very little phosphomannoseisomerase activity. The authors believe that mannose competitively inhibits both glucose and fructose phosphorylation and gives rise to an accumulation of mannose-6-phosphate.

It appears that the mannose toxicity occurring in honeybees can be considered a metabolic disease resulting from a lack of balance between hexokinase and phosphomannose-isomerase activity. Thus honeybees have developed metabolic schemes whereby they can handle large amounts of glucose or fructose, but they do not have a mechanism for converting mannose-6-phosphate to a utilizable form of carbohydrate. These investigators suggest that the effect of mannose in the honeybee is not widely different from that of galactose in patients with galactosemia, although of course different enzyme systems are involved.

Toxicity of Saturated Monobasic Aliphatic Acids and Their Esters

Technologic progress has increased the chances of man's exposure to many chemical substances. This is especially true of the aliphatic acids and their esters. Many of these compounds occur naturally in foods, and some may contaminate foods through their presence in insecticides, preservatives, solvents and dyes.

In two articles, W. F. von Oettingen (*A. M. A. Arch Indust. Health* **21,** *28, 100 (1960)*) has compiled extensive information about those acids with two to 18 carbons. The report is factual and encyclopedic in style. It describes the chemistry, uses and irritant action of each acid, its manner of absorption, fate and excretion, pharmacologic or toxic effect, pathologic changes, prophylaxis and treatment of the toxic action.

These papers provide an important and ready source of reference which should be available to nutritionists, chemists and physicians.

Physiologically Active Amines in Common Fruits and Vegetables

The content of serotonin and of catechol amines in bananas was reported recently (*Nutrition Reviews* **17,** *284 (1959)*). It was concluded that ingestion of large amounts

of this fruit could lead to abnormal quantities of 5-hydroxyindole acetic acid and catechol amines in the urine of man. Recently R. W. Ewer, J. A. Arkins, B. T. Heffernan and E. J. Lennon (*J. Clin. Endocrinol. Metab.* **19,** *1037 (1959)*) reported finding abnormal amounts of catechol amines in the urine of unselected hospitalized patients. Higher rates of excretion were found in patients with liver disease, infection of some types, psychotic reactions, or in those near death.

In further studies, fruits and vegetables were analyzed for their content of physiologically active amines (S. Undenfriend, W. Lovenberg and H. Sjoerdsma, *Arch. Biochem. Biophys.* **85,** *487 (1959)*). Each fruit or vegetable was homogenized, centrifuged and assayed for content of 5-hydroxytryptamine, tryptamine, tyramine and catechol amines.

Serotonin was found in high concentration in banana skin and in lesser amounts in the pulp. Green bananas contained less than did ripe ones. Pulp of plantain, a tropical fruit, contained even more serotonin than did that of bananas. Tomatoes, plums and avocados contained significant quantities.

Tryptamine was found in plums, and tyramine was present in bananas, avocados, oranges and plums. Dopamine was intensely concentrated in banana peels, but only small quantities were present in banana pulp and in avocados. Norepinephrine was distributed in a similar fashion being concentrated in banana peel. Small quantities were found in banana pulp and in potatoes.

Amino acid decarboxylation is not limited to a few plants, and may have important application since these substances could contribute to browning or discoloration of fruits. For example, certain amine oxidase inhibitors can prevent browning of banana skins.

The authors considered the presence of serotonin and catechol amines in these foods to be of no physiologic importance, since large quantities of these chemicals can be fed to man without adverse reactions. Apparently they are destroyed almost as rapidly as they are absorbed. The only clinical factor of significance would be the effect on urinary excretion of amines, which might lead to an erroneous diagnosis.

Dietary Aspects of Cardiovascular Disease

The Heart Disease Control Program of the Division of Special Health Services, Department of Health, Education and Welfare, Washington, D. C., has just issued a publication (PHS OM-1175) titled *Dietary Aspects of Cardiovascular Diseases, Selected References.* The publication has been prepared chiefly for health department professional personnel needing a guide to current information and teaching materials on this subject. It may be useful also to private physicians, professional personnel in voluntary health agencies and hospitals, research workers, and other professional groups.

Beside general information, references are given to material on calorie restriction (weight control and obesity), sodium restriction (congestive heart failure and hypertension), fat control (atherosclerosis and coronary artery disease), and to food composition tables in current use.

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The publication of NUTRITION REVIEWS has been undertaken to enable professionally trained people to keep abreast of current progress and to have available an unbiased, authoritative review of the current research literature in the science of nutrition.

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